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Transporters as information processors in bacterial signalling pathways

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Summary

Transporters are essential players in bacterial growth and survival, since they are key for uptake of nutrients on the one hand, and for defence against endogenous and environmental stresses on the other hand. Remarkably, in addition to their primary role in substrate translocation, it has become clear that some transporters have acquired a secondary function as sensors and information processors in signalling pathways. In this review, we describe recent advances in our understanding of the role of transporters in such signalling cascades, and discuss some of the emergent dynamic behaviour found in hallmark examples. A particular focus is placed on new insights into mechanistic details of information transfer between transporters and regulatory proteins. Quantitative considerations reveal that these signalling complexes can implement a remarkable diversity of regulatory logic functions, where the transporter can act as activity switch, as positive or negative reporter of transport flux, or as a signalling hub for the integration of multiple inputs. Such a dual use of transport proteins not only enables efficient substrate translocation but is also an elegant strategy to integrate important information about the cell's external conditions with its current physiological state.

Introduction

Active transporters are essential players in bacterial growth and survival, since they are key for uptake of essential nutrient on the one hand, and for defence against endogenous and environmental stresses, e.g. by export of toxic compounds, on the other hand (Fig. 1). This is reflected by the vast functional and mechanistic diversity in bacterial transport systems, which can be energised by ATP hydrolysis or the proton motif force, can act as importers or exporters, and can have broad substrate ranges or be highly specific (Padan, 2009; Wang *et al.*, 2009).

In addition to their primary role in substrate translocation, transporters can also possess a secondary function and serve as accessory components of sensory and signalling systems. In this role, they contribute to decision-making processes that enable the bacterial cell to adapt to changes in the prevailing environmental or intracellular conditions. For instance, transporters play a pivotal role in nutrient sensing, because many substrates first need to be actively translocated to the cytoplasm before a cytoplasmic sensor protein may detect the cue (Fig. 1). This is frequently the case in inducible carbohydrate utilization systems, such as the arabinose and lactose systems of *Escherichia coli* (Müller-Hill, 1996; Schleif, 2000). The importers for these carbon sources are constitutively produced at a low level to allow a basal rate of uptake. Only when this results in accumulation of elevated intracellular substrate concentrations can these be detected by cytoplasmic regulators, which in turn activate expression of genes required for increased rates of import and metabolism of the available carbon source (Megerle *et al.*, 2008; Fritz *et al.*, 2014). In cells lacking the transporter, the substrate cannot enter the cytoplasm, preventing detection and the resulting cellular response (Daruwalla *et al.*, 1981).

While this example may be viewed as an indirect role of transporters in substrate perception, it is becoming increasingly recognized that some transporters have acquired *bona fide* signalling functions. These so-called 'trigger transporters' can directly bind to and communicate with bacterial signalling pathways, such as one-component (1CS) and two-component (2CS) regulatory systems (Tetsch and Jung, 2009; Unden *et al.*, 2016). In this way, the cell elegantly combines the exquisitely sensitive ability of transporters to bind and report on substrate availability with the regulatory power of 1CS and 2CS to translate this information into an appropriate cellular response. The information relayed from transporter to regulatory system may be the external or internal concentration of a ligand, or it may be the transport activity itself (Fig. 1). Interestingly, in some cases the transporters have even lost their primary substrate-translocating function and only retained their role as ligand binding `scaffolds' reporting on extracellular substrate concentrations. In the Uhp system of *E. coli*, for instance, the UhpC transporter displays only marginal transport activity for its substrate, glucose 6-phosphate (G6P), but faithfully detects and signals the presence of G6P to the UhpB/UhpA 2CS, which in turn activates the production of the hexose phosphate transporter UhpT (Island and Kadner, 1993; Schwöppe *et al.*, 2003).

In this MicroReview we will discuss the role of active transporters as accessory or sensory components of signalling pathways. We will address systems in which the transporter functions as activity switch, as positive or negative reporter of transport flux as well as systems in which transporters mediate the integration of multiple signals. Based on recent advances in our molecular and quantitative understanding of such signalling pathways, we provide a descriptive theoretical framework for their classification according to system behaviour, which will aid researchers in the characterisation of newly identified sensory transporters.

Transporters as activity switches

Uptake and metabolism of C_4-dicarboxylates, such as fumarate or malate, in many bacteria is regulated by a 2CS that is under negative control of secondary C_4-dicarboxylate transporters (Unden *et al.*, 2016). In *E. coli*, this 2CS is comprised of the histidine kinase DcuS and the response regulator DcuR. Under aerobic conditions, fumarate and other C_4-dicarboxylates are taken up by the secondary transporter DctA, and expression of the *dctA* gene and fumarate metabolic genes is controlled by DcuS-DcuR. Under anaerobic conditions, fumarate can be used as electron acceptor for fumarate respiration, leading to production of succinate. Here, uptake occurs via the fumarate/succinate antiporter DcuB, and expression of *dctB* and genes required for fumarate respiration is again controlled by DcuS-DcuR. These processes have been recently reviewed in detail (Unden *et al.*, 2016). Interestingly, DcuS itself is fully capable of binding suitable substrates (Kneuper *et al.*, 2005; Monzel and Unden, 2015), but is only able to respond to such binding events in the presence of either DctA or DcuB. In the absence of transporters, the kinase adopts a constitutive 'ON' state and fails to respond to stimuli (Kleefeld *et al.*, 2009; Witan *et al.*, 2012; Unden *et al.*, 2016). The role of the transporters in C_4-dicarboxylate sensing appears to be that of an activity switch, transferring the kinase from the constitutively active state to a substrate-responsive state (Fig. 2A).

DcuS possesses an extracellular PAS domain flanked by two transmembrane helices, which serves as the ligand binding site for C_4-dicarboxylates or citrate (Kneuper *et al.*, 2005; Unden *et al.*, 2016). The cytoplasmic part of DcuS is composed of a second PAS domain, followed by the kinase domains. Activation of DcuS occurs by a piston-like movement of

102 transmembrane helix 2 by four amino acids towards the periplasmic side, most likely caused
103 by conformational changes in the periplasmic PAS domain upon ligand binding (Monzel and
104 Unden, 2015). Under aerobic conditions, the transporter DctA forms a complex with DcuS,
105 mediated by direct protein-protein interactions between an amphipathic cytoplasmic helix
106 near the C-terminus of DctA and the cytoplasmic PAS domain of DcuS (Witan *et al.*, 2012).
107 Several lines of evidence suggest that formation of this complex is sufficient to bring DcuS
108 into its substrate-responsive state, and that neither transport nor substrate binding by DctA
109 (or DcuB) play any direct role in the signalling process. Firstly, mutations in the C_4-
110 dicarboxylate binding site of DctA abolish transport, but do not affect signalling by DcuS
111 (Steinmetz *et al.*, 2014). Similarly, transport-defective DcuB variants have been constructed
112 that maintain their ability to switch DcuS to the responsive state (Kleefeld *et al.*, 2009).
113 Secondly, DcuS can be activated by citrate, which is not a substrate for DctA (Steinmetz *et*
114 *al.*, 2014). And thirdly, the substrate concentrations required for transport and signalling
115 differ by several orders of magnitude. Uptake of fumarate by *E. coli*, presumably catalysed
116 by DctA, occurs with an apparent K_m of 30 µM (Kay and Kornberg, 1971), compared to an
117 apparent K_m of 2-3 mM for activation of DcuS-dependent promoters (Kneuper *et al.*, 2005).
118 Thus, maximal rates of transport are already supported by substrate concentrations that are
119 insufficient to fully induce DcuS (Unden *et al.*, 2016). DctA therefore clearly acts as an
120 activity switch for DcuS and does not contribute any additional sensory functions to the
121 signalling process. It was recently shown that a functionally equivalent complex is formed
122 between DcuS and DcuB under anaerobic conditions (Wörner *et al.*, 2016).

123 At a first glance, it does not appear clear what advantages such a regulatory strategy
124 imparts to the cell. Why employ a transporter in a signalling pathway when neither
125 substrate binding nor transport are factored into the regulatory decision taken by the cell?

126 The citrate-responsive kinase CitA of *E. coli*, which is closely related to DcuS, does not
127 require a transporter to respond to its substrate, showing that these kinases can function on
128 their own (Scheu *et al.*, 2012). Intriguingly, the dual use of DctA as both a signalling
129 modulator of DcuS and high-affinity uptake system for C_4-dicarboxylates causes the DcuS-
130 DcuR system to respond in a two-mode fashion (Unden *et al.*, 2016) (Fig. 2A). When no or
131 only micromolar concentrations of C_4-dicarboxylates are present, DcuS will be present in the
132 ligand-free state. Its activity under these conditions will only depend on the presence of
133 DctA. If insufficient amounts of DctA are present to occupy all DcuS proteins, any free kinase
134 molecules will be constitutively active, leading to increased production of DctA. This
135 response is switched off as soon as DcuS is saturated with DctA (Steinmetz *et al.*, 2014). In
136 this mode, termed Mode II (Unden *et al.*, 2016), the input information is 'concentration of
137 DctA', thereby leading to a negative autoregulation of transporter levels (Fig. 2B). The
138 physiological role of keeping DctA at such an equilibrium may be to ensure homeostatic
139 control of the transport capacity for C_4-dicarboxylates. The high affinity of the transporter
140 allows uptake even at low substrate concentrations, enabling the cell to utilise these in
141 anabolic or catabolic metabolism (Unden *et al.*, 2016). In Mode I, DcuS is saturated with
142 DctA and therefore fully switched to its substrate-responsive state (Unden *et al.*, 2016) (Fig.
143 2A). The sole input information here is 'concentration of substrate' (Steinmetz *et al.*, 2014),
144 but high (millimolar) concentrations of the substrate are required to elicit a strong response
145 (Kneuper *et al.*, 2005) (Fig. 2B). The low substrate affinity of DcuS may ensure that the cell
146 does not commit to a costly induction of C_4-dicarboxylate utilization systems unless
147 sufficiently high concentrations are available to support growth on these substrates as
148 primary carbon sources. Hence, the qualitative model of signal integration by the DcuS-DcuR
149 system (Fig. 2A) seems to implement the regulatory logic of an OR gate, which either

becomes activated by high external C_4-dicarboxylate levels *or* by a shortage of transporters within the cell. This OR-gate logic can be nicely illustrated with the aid of a mathematical model for the DcuS/DctA/C_4-dicarboxylate interaction scheme depicted in Figure 2A: If one assumes binding of DctA to DcuS (Mode II binding with binding constant K_2) and cooperative binding of C_4-dicarboxylates to the DctA-DcuS complex (Mode I binding with binding constant K_1 and Hill coefficient n), then the active fraction of the histidine kinase, f_{ON}, (i.e. the sum of the unbound DcuS fraction and DcuS-DctA-C_4-dicarboxylate complex) reads

$$f_{ON} = \frac{1 + \frac{[A][C]^n}{K_2 K_1^n}}{1 + \frac{[A]}{K_2} + \frac{[A][C]^n}{K_2 K_1^n}}, \qquad (1)$$

where $[A]$ and $[C]$ are the concentrations of DctA and C_4-dicarboxylates, respectively. Note that this mathematical model not only resembles the regulatory logic of an OR gate (Fig. 2B), as expected qualitatively, but is also in good quantitative agreement with the experimental data by (Steinmetz *et al.*, 2014). This underlines that the simple reaction scheme depicted in Figure 2A is indeed sufficient to describe this elegant way of signal integration.

Sensors of transport flux

Although in the previous example the DctA transporter is a fully functional importer for C_4-dicarboxylates, transport activity *per se* is dispensable for signalling. There are, however, an increasing number of systems in which substrate translocation by the transporter is essential for stimulus perception via the signalling system. Based on this strict dependence on transport flux, we will in the following refer to such tandems of transporters and

signalling systems as “flux sensors”. Moreover, we will distinguish between two classes of flux sensors: On the one hand, we define positive flux sensors as those in which a high transport activity acts as cue for the activation of signalling, while on the other hand, in negative flux sensors signalling is activated at a low transport activity. Accordingly, mutants deleted for the respective transporters also show opposing behaviour of the signalling systems: In positive flux sensors the signalling system turns to its default ‘OFF’ state in the absence of the transporter, while in negative flux sensors the signalling system remains locked in the default ‘ON’ state.

Positive flux sensors

The paradigm example for a positive flux sensor is the Bce system of *Bacillus subtilis*, which provides resistance against the peptide antibiotic bacitracin and a few other antimicrobial peptides (Fig. 3A). The system consists of the ABC-transporter BceAB that mediates the actual resistance, and the 2CS BceRS that controls expression of the transporter operon (*bceAB*) (Ohki *et al.*, 2003). The most striking characteristic of this system is that the BceS kinase lacks any obvious sensory domains. Instead BceS completely depends on the transporter for detection of substrate peptides and deletion of the transporter abolishes any induction of the target promoter, P_{bceA} (Rietkötter *et al.*, 2008), highlighting that the default state of the 2CS is ‘OFF’. BceAB therefore not only acts as resistance determinant, but also as primary sensor of the antibiotic. Interestingly, resistance and signalling can be genetically separated by single amino acid substitutions in the transport permease BceB, showing that BceAB truly is a dual-function transporter (Kallenberg *et al.*, 2013). Biochemical analysis of the proteins involved showed that the kinase and transporter form a

194 sensory complex in the cytoplasmic membrane of the cell, and that the transporter can
195 directly influence kinase activity (Dintner *et al.*, 2014). The mechanistic basis of signal
196 transmission between BceB and BceS, however, remains unknown. Mutagenesis of the
197 ATPase component, BceA, further demonstrated that ATP hydrolysis is required for
198 activation of BceS, suggesting that transport *activity* is essential for the signalling process,
199 rather than mere ligand binding (Rietkötter *et al.*, 2008). Mathematical modelling of the
200 quantitative response dynamics of the Bce system provided evidence that it directly
201 responds to transport activity of BceAB, with each BceS kinase measuring the antibiotic flux
202 experienced by the transporter with which it forms a sensory complex (Fritz *et al.*, 2015)
203 (Fig. 3B).

204 Our current understanding of the Bce system has led to the following model of its biological
205 function (Fig. 3B and C). In un-challenged cells, basal promoter activity of the *bceAB* operon
206 produces a small number of transporters that can form sensory complexes with BceS, which
207 itself is produced from a constitutive promoter. Bacitracin and other antimicrobial peptides
208 bind to intermediates of the lipid II cycle of cell wall biosynthesis (Breukink and de Kruijff,
209 2006; Economou *et al.*, 2013). How transport can mediate resistance against an antibiotic
210 targeting a surface structure of the cell is currently unknown, but presumably involves
211 removal of the antibiotic from the target, freeing the latter for access by cell wall
212 biosynthetic enzymes. Such a mechanism would be similar to that observed for self-
213 resistance in some producer organisms of antimicrobial peptides (Stein *et al.*, 2003; Okuda
214 *et al.*, 2008). In this case, the complexes between the antibiotic and its target molecule form
215 the substrate for the BceAB transporter. The higher the concentration of the antibiotic, the
216 more antibiotic-target complexes are formed, and the higher is the transport activity of
217 BceAB. This increased activity is directly detected by the BceS kinase, presumably through

physical contact to the transporter, and leads to activation of signalling and increased production of the transporter (cf. Fig. 3B; *red arrow*). The resulting additional capacity for transport will keep the cellular target free of antibiotic, thereby reducing the concentration of substrates for individual transporters. The decreased transport activity experienced by individual BceAB molecules then in turn swiftly turns off signalling via BceS (cf. Fig. 3B; *green arrow*). This flux-sensing mechanism thus implements a negative feedback on regulation, allowing the cell to quickly establish a new steady-state level of transporter numbers. Experimentally observed response times were indeed very fast with equilibrium reached after approximately one third to one half of the generation time (Fritz *et al.*, 2015). Importantly, by using the transporter as both activator of signalling and remover of its own substrate, the cell precisely adapts the new steady-state level of transporters depending directly on the cell's capacity for detoxification, not merely the present concentration of the antibiotic. The adaptive power of this regulatory strategy is highlighted by a very large input-dynamic range, allowing a gradual response over nearly three orders of magnitude of antibiotic concentrations, which is considered to be more cost-effective than a simple dose-dependent response to antibiotic concentrations (Fritz *et al.*, 2015).

Phylogenetic analyses of Bce-like systems revealed that they are found throughout the low G+C content Gram-positive bacteria, but not outside of this group (Joseph *et al.*, 2002; Dintner *et al.*, 2011). BceAB-like transporters are almost always found together with BceRS-like 2CS, and their transport permeases and histidine kinases have co-evolved, suggesting a general functional inter-dependence between them (Dintner *et al.*, 2011). All systems that have been studied experimentally to date are involved in resistance against antimicrobial peptides, so not only the regulatory principle but also their physiological role seems to be conserved (Dintner *et al.*, 2011; Gebhard and Mascher, 2011; Gebhard, 2012; Revilla-

242 Guarinos *et al.*, 2014). In some bacteria, variations on the theme described above for the
243 Bce-system in *B. subtilis* exist. Whereas in the Bce-system the same transporter fulfils the
244 roles of resistance determinant and antibiotic sensor, these functions are divided between
245 two separate transporters in *Staphylococcus aureus* (Hiron *et al.*, 2011) and *Enterococcus*
246 *faecalis* (Gebhard *et al.*, 2014). Whether this is an evolutionary intermediate, e.g. following
247 loss of the 2CS for one of the transporters, or gain of an additional orphan transporter, or
248 whether such a regulatory strategy gives the cell a particular advantage remains unclear.
249 Interestingly, two systems have been described where the 2CS is activated by a sensory
250 BceAB-like transporter, but the actual resistance is then mediated by induction of entirely
251 unrelated genes, such as the *dltABCD* operon (encoding for D-alanylation of teichoic acids)
252 and *mprF* (for L-lysinylation of phospholipids). One example for this is the nisin resistance
253 system 'module 12' of *Lactobacillus casei* (Revilla-Guarinos *et al.*, 2013). The second
254 example is comprised of the 2CS GraSR and transporter VraFG of *S. aureus*, where
255 communication between transporter and kinase appears to involve a fifth protein, GraX
256 (Falord *et al.*, 2012). The VraFG transporter does not have the ability to confer resistance
257 against antimicrobial peptides on its own (Falord *et al.*, 2012). In this case, the negative
258 feedback that governs the response dynamics of the *B. subtilis* Bce-system appears to be
259 missing. It would be interesting to investigate the effects of this on gene regulation, or if
260 induction of *dltABCD* and *mprF*, which together reduce access of the antibiotics to their
261 targets (Revilla-Guarinos *et al.*, 2014), could have an effect equivalent to active removal of
262 the antibiotic.
263
264 While in the Bce system discussed above, the transporter interacts via its permease domain
265 with the 2CS to implement a flux sensor, also other components of multi-partite

transporters were described to interact with signalling systems. For instance, in *E. coli* the periplasmic maltose binding protein MalE interacts with the Tar chemoreceptors in the presence of maltose, and thereby modulates chemotaxic behaviour (Hazelbauer, 1975; Zhang *et al.*, 1999). Similarly, in *Agrobacterium tumefaciens* the chromosomal virulence gene E (*chvE*) encodes a periplasmic binding protein that binds several neutral sugars and sugar acids, and subsequently interacts with the VirAG 2CS to stimulate virulence gene expression (Hu *et al.*, 2013).

Another well-studied example is the citrate uptake system of *Bordetella pertussis*, in which the tripartite tricarboxylate transporter (TTT) BctCAB interacts via its periplasmic binding protein BctC with the histidine kinase BctE (Antoine *et al.*, 2003) (Fig. 4). Here, citrate binding to BctC not only enables citrate uptake through BctAB, but also activates the 2CS BctDE, which in turn triggers expression of the *bctCAB* transporter operon (Antoine *et al.*, 2005). Intriguingly, disruption of *bctA* was shown to abolish citrate uptake by *B. pertussis* (Antoine *et al.*, 2003), while signalling through BctDE was highly elevated in this mutant (Antoine *et al.*, 2005). This clearly shows that not active citrate transport by BctCAB, but rather accumulating levels of citrate-bound BctC molecules are the cue detected by the 2CS BctDE. It is nevertheless conceivable that the fully functional (wild-type) Bct system may function akin to a positive flux sensor: qualitatively, one would expect that the rate of citrate uptake is directly proportional to the level of citrate-bound BctC molecules. Because these citrate-BctC complexes at the same time serve as the input to the signalling system, citrate influx should be proportional to signalling. To understand the quantitative implications of this signalling strategy, it will be important to consider the relative affinities of substrate-bound BctC to the histidine kinase, BctE on the one hand, and the transport permease components BctAB on the other hand. Preferential binding of BctC to one of its

290 partners could have drastic effects on the relationship between transport flux and signalling.
291 Therefore, more quantitative experiments and mathematical modelling are needed to fully
292 understand the regulatory implications of linking transport to signalling in this way.
293 The Bct system of *B. pertussis* is homologous to the paradigm TTT transporter TctCAB of
294 *Salmonella typhimurium*, which is likewise controlled by a 2CS, TctE-TctD (Widenhorn *et al.*,
295 1989). It is, however, not known whether TctC plays a similar role in signalling as in the *B.*
296 *pertussis* system. Intriguingly, *B. subtilis* possesses an orphan homologue to BctC, named
297 YflP, which is genetically associated with a 2CS, YflR-YlfQ. Although it remains to be proven
298 experimentally, it has been proposed, based on gene arrangement and the absence of the
299 transmembrane components of the transporter, that YflP may have a sensory role in this
300 system (Winnen *et al.*, 2003). This suggestion raises the question as to the original function
301 of BctC-like binding proteins, substrate binding for transport or as an accessory component
302 of signalling systems. A phylogenetic analysis of TTT systems revealed that the larger
303 transmembrane component, the TctA-like proteins, are often found alone and are most
304 widely distributed, even including Archaea (Winnen *et al.*, 2003). They are only occasionally
305 found together with TctB- and TctC-homologues, and the full set of proteins only occurs in
306 bacteria. This has been interpreted as TctA being the actual transporter in its ancestral form
307 that later acquired the smaller accessory membrane protein TctB and the periplasmic
308 binding protein TctC, e.g. to modify function or increase substrate affinity (Winnen *et al.*,
309 2003). It is therefore conceivable that TctC-like proteins originated as signalling proteins and
310 were later co-opted into transport systems. It would be highly interesting to test if the
311 regulatory principle so far only shown for the *B. pertussis* Bct system holds true for its
312 homologues and if orphan TctC-like proteins truly possess signalling activities.
313

Negative flux sensors.

One of the best-understood examples for a negative flux sensor is the regulation of the phosphate starvation response of *E. coli*. Here, the 2CS PhoR-PhoB forms a sensory complex with the ATP-binding cassette (ABC) transporter $PstSCAB_2$, mediated by the chaperone-like protein PhoU (reviewed in (Hsieh and Wanner, 2010)). Phosphate-limitation activates signalling by PhoR-PhoB and leads to the induction of the entire Pho-regulon, which includes genes encoding the PhoR-PhoB 2CS, the $PstSCAB_2$ transporter, PhoU and several transporters and enzymes involved in exploiting alternative phosphorus sources (Wanner, 1993; van Veen, 1997). Through the resulting changes, the cell increases both its capacity for phosphate uptake and the availability of free phosphate. In the absence of $PstSCAB_2$ or PhoU, the kinase PhoR is in a constitutively activated state, leading to full induction of the Pho-regulon, irrespective of phosphate availability. Thus, the default state of the 2CS is 'ON', and the transporter acts as repressor of signalling under phosphate-replete conditions. This functional interdependence of 2CS, $PstSCAB_2$ and PhoU has not only been found in *E. coli*, but also in *Sinorhizobium meliloti* (Yuan *et al.*, 2006) and *Mycobacterium smegmatis* (Gebhard and Cook, 2008). Interestingly, in *Bacillus subtilis* the transporter does not appear to be involved in phosphate-signalling (Qi *et al.*, 1997), showing that the regulatory principle is wide-spread but not universal.

The PhoR histidine kinase is devoid of any apparent extracellular sensory domains, but contains a cytoplasmic PAS (Per-ARNT-Sim) domain harbouring the interaction interface with PhoU (Gardner *et al.*, 2014; Gardner *et al.*, 2015). PhoU also interacts with the ATPase component of the transporter, PstB, and therefore appears to be the hub of the seven-protein signalling complex (Gardner *et al.*, 2014) (Fig. 5). Signalling is thought to occur by the

following mechanism. Under high phosphate conditions, the $PstSCAB_2$ transporter is fully occupied and all transporters are switched to the 'transport active' state. In this state, the transporter is also 'signalling active' and represses the kinase activity of PhoR-PhoB while the phosphatase activity dominates (Hsieh and Wanner, 2010) (Fig. 5A). When phosphate becomes limiting, not all transporters are active at all times, and those that temporarily become inactive lose their repressive function, leading to the activation of signalling (Fig. 5B). According to this model, a cell experiencing phosphate-limitation possesses a mixture of transport-active and inactive copies of $PstSCAB_2$. At any given time, the currently inactive copies promote signalling and cause activation of the Pho-regulon, while the active copies import phosphate and allow the cell to continue growing (Hsieh and Wanner, 2010). In this way, the cell responds not simply to the external concentration of phosphate, but rather to its ability to scavenge this essential nutrient, which ultimately is the more relevant parameter for survival.

In addition to increasing the number of transporters as a response to phosphate-limitation, the cell also produces enzymes such as alkaline phosphatases to liberate phosphate from other sources (Wanner, 1993; van Veen, 1997). This will increase the external concentration of free phosphate, raise the intake flux per $PstSCAB_2$ transporter and ultimately shut down the response (Fig. 5C).

Notably, upon phosphate starvation the cell not only responds by increasing the copy number of $PstSCAB_2$ transporters, but also up-regulates the production of the PhoR-PhoB 2CS in an auto-activating positive feedback loop (Hoffer *et al.*, 2001) (Fig. 5C)1. In principle, positive feedback can lead to switch-like or even hysteretic behaviour, where the response to a current stimulus depends on the previous history of stimuli (Fritz *et al.*, 2007; Lambert

360 *et al.*, 2014). Indeed auto-amplification of the PhoR-PhoB 2CS has been attributed with
361 "learning" behaviour, because phosphate-starved cells displayed a significantly faster
362 induction of the Pho response than non-starved cells - even after 2 hours of growth in
363 phosphate-containing medium (Hoffer *et al.*, 2001). While the physiological role of such
364 priming behaviour is still elusive, clear selective advantages may arise if fluctuations in
365 environmental phosphate availability are correlated. For instance, if one period of
366 phosphate starvation is indicative of subsequent starvation periods, it might be beneficial to
367 keep memory on previous starvation responses.

368 Not much is known about the molecular mechanism by which the transporter and PhoU
369 repress PhoR activation. One hypothesis is that PhoU somehow transmits conformational
370 changes as part of the transport cycle of $PstSCAB_2$ to the kinase (Gardner *et al.*, 2014).
371 Importantly, transport and signalling are genetically separable functions of $PstSCAB_2$, as
372 amino acid substitutions have been identified in the permease component PstC that abolish
373 transport but still allow repression of PhoR (Cox *et al.*, 1989). In contrast, mutations that
374 prevent ATP-hydrolysis by the ATPase PstB caused defects in both transport and signalling
375 (Cox *et al.*, 1989). Considering that PhoU interacts with the PstB subunit of the transporter
376 (Gardner *et al.*, 2014; Gardner *et al.*, 2015), it therefore appears likely that the information
377 transmitted to the kinase is derived from the ATPase activity of the transporter, i.e. as long
378 as the ATPase hydrolyses ATP, PhoU represses PhoR. Two possibilities of how this may be
379 accomplished have been discussed (Gardner *et al.*, 2014): If the complex between the 2CS,
380 PhoU and $PstSCAB_2$ is stable regardless of the phosphate concentration, then PhoU may
381 have the ability to cause PhoR to switch between active and inactive states depending on
382 $PstSCAB_2$ activity. Alternatively, the complex may dissociate when the transporter is inactive
383 to allow the kinase to adopt its default active state.

Interestingly, it appears that PhoU plays a second important role in controlling not only the start of the Pho response, but also its termination (Fig. 5C). When a low-phosphate adapted cell returns to phosphate-replete conditions, repression of PhoR-PhoB has to be restored to terminate the response, as discussed above. The high levels of $PstSCAB_2$ and PhoU that were produced during the phosphate-limited period ensure that the 2CS is quickly returned to its repressed state. However, the presence of high transporter levels also poses a risk to the cell, as it could lead to the accumulation of toxic intracellular phosphate concentrations (Rice *et al.*, 2009). This appears to be prevented by PhoU, which has the ability to inhibit phosphate uptake by $PstSCAB_2$ when intracellular phosphate concentrations are high (Rice *et al.*, 2009). Similar negative effects of PhoU on phosphate uptake have also been observed in *Pseudomonas aeruginosa* (de Almeida *et al.*, 2015) and *Caulobacter crescentus* (Lubin *et al.*, 2016). This second regulatory function of PhoU thus constitutes an elegant mechanism to immediately curb uptake by the transporter and thereby prevent accumulation of toxic intracellular phosphate when the external availability of phosphate suddenly increases. Detailed investigations of the multiple activities of this enigmatic hub-protein will be essential to fully understand how phosphate transport connects to signalling and how these processes are embedded in the physiology of the cell.

Transporters involved in signal integration

In the systems discussed above, a unique stimulus is responsible for sensing and signalling. Either the transporter level, the transporter activity (flux) or the 'result' of transport, namely the internal substrate, is detected. Yet many microbial adaptation processes not only rely on a single source of environmental information, but instead require the combinatorial

integration of multiple external and internal cues. However, the sensors involved in stimulus perception are subject to biochemical constraints, which limit the number cues that can be detected by a single protein. Signal integration is therefore often achieved by interactions between multiple sensory systems, which can also incorporate transporters as sensors of substrate availability and/or reporters of transport flux.

One of the best-studied systems that integrates two sources of input information is the Mal-system required for maltose-uptake in *E. coli* (Fig. 6). Here, the import of maltose and maltodextrins depends on the ABC-transporter $MalEFGK_2$, which is genetically organized in two operons, *malEFG* and *malK-lamB-malM* [reviewed, e.g., in (Boos and Shuman, 1998)]. Both operons are regulated by the transcriptional activator MalT (Hofnung *et al.*, 1974; Hofnung, 1974). Interestingly, activation of MalT itself depends on two distinct inputs. Firstly, in the absence of external maltodextrins the ATP binding subunits of the transporter, $MalK_2$, sequester MalT into an inactive (ADP-bound) form (Schreiber *et al.*, 2000; Joly *et al.*, 2004) and thereby prevent its activation (Panagiotidis *et al.*, 1998). Only if $MalEFGK_2$ actively imports maltodextrins into the cell, ATP hydrolysis by $MalK_2$ triggers release of MalT and allows the latter to assume its active, ATP-bound form (Panagiotidis *et al.*, 1998; Schreiber *et al.*, 2000). Secondly, ATP-bound MalT is then further stabilized by internal maltotriose – an intermediate product of maltodextrin metabolism (Raibaud and Richet, 1987) – which triggers full activation of MalT (Schreiber *et al.*, 2000; Joly *et al.*, 2004) . Of note, this second mode of sensing is very similar to internal substrate sensing in the Lac-system of *E. coli*, in which allolactose – an intermediate product of lactose metabolism – is detected by the *lac* repressor LacI (Müller-Hill, 1996).

By using such a dual-input sensory strategy, the cell up-regulates the production of maltose transporters only if (*i*) there is a sufficient flux of maltodextrins into the cell and if (*ii*) internal substrate has accumulated to significant levels. Using such an AND gate, the cell asserts that physiological levels of endogenously produced maltotriose, e.g. during metabolism of glycogen (Ehrmann and Boos, 1987), do not cause the futile production of transporters in the absence of external substrate. This raises the question as to why MalT should act as an internal sensor of maltotriose at all, considering that the sequestration of MalT by inactive transporters already appears to act as a functional flux sensor reporting on external maltose availability. One potential benefit of this dual mode of sensing might be that internal substrate sensing can easily implement a positive feedback loop on regulation: increased production of transporters raises the rate of substrate accumulation, which in turn further up-regulates transporter production. In the classical lactose and arabinose utilization systems, this positive feedback was shown to trigger switch-like `all-or-none' behaviour at intermediate inducer concentrations, with one subpopulation of cells fully committing to sugar utilization and a second subpopulation not investing into costly synthesis of the associated metabolic program at all (Ozbudak *et al.*, 2004; Megerle *et al.*, 2008; Fritz *et al.*, 2014). Such a diversification of a phenotypic trait within a genetically identical population may serve as a `bet-hedging' strategy in fluctuating environments, in which sugars may come and go at unpredictable times (Veening *et al.*, 2008). Nevertheless, in theory, a similar positive feedback could be implemented with a flux-sensor alone, if for instance the regulator MalT were to be up-regulated in response to influx of maltose, leading to an increase in free, i.e. active pool of MalT. However *malT* is not subject to positive auto-regulation (Debarbouille and Schwartz, 1979; Chapon, 1982; Decker *et al.*, 1998), suggesting that this hypothetical scenario is not implemented in the Mal-system.

Thus, it seems likely that the need for positive feedback-induced heterogeneity cannot be the only factor that shaped the evolution of this dual mode of sensing. Further quantitative experimental and theoretical work is needed to shed light on the precise response-dynamics of this system.

A remarkable example for a system integrating three physiological stimuli is the Cad system, which is involved in the acid tolerance response of *E. coli* (Foster, 2004) (Fig. 7A). Upon exposure to acidic pH and in the presence of lysine, *E. coli* strongly up-regulates the expression of the *cadBA* operon, encoding the lysine decarboxylase CadA and the lysine/cadaverine antiporter CadB (Meng and Bennett, 1992) (Fig. 7B). Together, they increase both the intra- and extracellular pH via consumption of a cytoplasmic proton during the decarboxylation of lysine to cadaverine, followed by the excretion of the more basic cadaverine in exchange for a lysine molecule. Ultimately, the accumulation of external cadaverine turns off the expression of *cadBA*, leading to a transient Cad response (Neely and Olson, 1996; Fritz *et al.*, 2009) (Fig. 7B).

Interestingly, signal integration is achieved by the membrane-integrated ToxR-like 1CS CadC, which is able to sense low pH (Tetsch *et al.*, 2011; Buchner *et al.*, 2015) and high cadaverine levels (Eichinger *et al.*, 2011; Haneburger *et al.*, 2012) via its periplasmic domain, while its cytoplasmic domain directly binds to and activates the *cadBA* promoter (Kuper and Jung, 2005). However, CadC cannot directly sense the presence of lysine (Tetsch *et al.*, 2008), and instead relies on the secondary lysine/H^+ symporter LysP, which is required for lysine uptake from the growth medium. Early experiments showed that *lysP* mutants lead to lysine-independent *cadBA* expression (Popkin and Maas, 1980), while LysP overproduction caused repressed *cadBA* activity (Neely *et al.*, 1994), suggesting that LysP is a negative regulator of

CadC activity in the absence of lysine. Recently, it was shown that at low external lysine concentrations LysP indeed forms a complex with CadC via intramembrane and periplasmic contacts (Rauschmeier *et al.*, 2014), which keep CadC in an inactive state – regardless of external pH. Only if LysP imports lysine at sufficiently high rates, lysine-dependent conformational changes in LysP transduce the lysine signal via a direct conformational coupling to CadC without resolving the interaction completely (Rauschmeier *et al.*, 2014). Hence, by integrating the transport activity of LysP into the decision for activating the Cad response, the cell asserts that the acid defence via lysine decarboxylation is only activated if the substrate for the reaction – lysine – is actually present in the environment.

Concluding remarks

During the course of evolution bacterial transporters developed into sophisticated molecular machines with exquisite abilities to bind their cognate substrates. As highlighted in this review, this not only enables efficient substrate translocation, but can also be used to provide the cell with important information about its extra- and/or intracellular state. This is achieved via direct or indirect contacts between the transporter and a membrane-associated signalling protein. Depending on the regulatory logic of these interactions, a vast range of physiological responses can be implemented, ranging from activity switches, positive and negative flux sensors to multi-input signalling hubs. While the mechanistic details of signal transfer at the interface between transporter and signalling protein are just beginning to be revealed, in many systems the transporter regions or even specific amino acids involved in transport are distinct from those used for information transfer. Given such a modular structure, it seems likely that these two traits either evolved independently of

each other, or at least functionally diverged over the course of evolutionary time. This, together with the large diversity of implemented signalling behaviours, provokes the question as to why transporters are not observed more commonly as (co-)sensors in nature. One potential reason for this might be that the key experiments revealing transporters as essential for stimulus perception are in fact not necessarily intuitive. They require the activity of a target promoter under investigation to be monitored in a transport-deficient mutant – a scenario that is not routinely assayed when characterising a transporter or signalling system. Therefore, one might speculate that regulatory roles of transporters are far more common than currently known. As the existence and physiological relevance of information-processing transporters is becoming increasingly recognised, systematic screens should help identify other examples. Subsequent quantitative characterisation in conjunction with mathematical modelling can then reveal the actual logic implemented in each respective system and may even lead to the discovery of further regulatory complexity and novel signalling strategies in bacteria.

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Figure legends

Figure 1. Transporters as information processors in bacterial cells. The optimization of bacterial growth and survival critically relies on active transporters (*green*) responsible for uptake of essential nutrients (*purple*) and export of endogenous and environmental stressors (*red*). In order to adapt their transport capacity to changes in prevailing conditions, transporters frequently play pivotal roles in signalling (*red wave arrows*) to regulatory systems (*orange)* that control the expression of transporter genes (*straight red arrows*). These roles range from transporters as sensors of external substrate level, to transporters as indirect mediators for internal substrate detection, to transporters serving as *bona fide* flux sensors that report on their transport activity *per se*.

Figure 2. The transporter DctA acts as activity switch on the histidine kinase DcuS during regulation of C_4-dicarboxylate utilization in *E. coli*. (A) The C_4-dicarboxylate transporter DctA (*green*) is not only responsible for high-affinity uptake of C_4-dicarboxylates (*purple*), but also involved in regulating its own production. In the absence of DctA, the kinase DcuS is constitutively active (*left*) and up-regulates transporter production (*dashed arrow*). Binding of DctA to DcuS sequesters DcuS to an 'OFF'-state (*centre*), leading to a negative feedback of DctA on its own production. This is referred to as Mode II and serves as sensor of DctA levels. In complex with DctA, DcuS is switched to a substrate-sensitive state, in which the histidine kinase is activated by binding of C_4-dicarboxylates (*right*; referred to as Mode I). **(B)** A simple mathematical model derived from the reaction scheme in panel A illustrates the dual modes of sensing and is compatible with quantitative experimental data published previously (Steinmetz *et al.*, 2014). Within the model, DcuS activity is proportional to the

773 probability of finding DcuS in an 'ON' conformation, i.e. either unbound or in a DcuS-DctA-
774 C_4-dicarboxylate complex, as given by Equation (1) in the main text. The graph was
775 generated using the following parameters: K_1 = 0.5 mM, K_2 = 10 nM and n = 2.

776

777 **Figure 3. In *B. subtilis*, a sensory complex of the histidine kinase BceS and the ABC**
778 **transporter BceAB serves as a positive flux sensor. (A)** The ATP-binding cassette
779 transporter BceAB (*green*) confers resistance against the antimicrobial peptide bacitracin
780 (Bac, *red*) by removing the latter from its cellular target undecaprenyl pyrophosphate (UPP,
781 *yellow*). A complex between the 2CS BceRS (*orange*) and BceAB serves as a sensor of
782 transport activity ('flux-sensing'), which stimulates expression of the *bceAB* operon in
783 response to bacitracin stress. For simplicity, BceS is represented as a monomer instead of
784 the biologically active dimer. **(B)** Mathematical simulations of a previously published model
785 for the Bce system (Fritz *et al.*, 2015) show the long-term activity of the P_{bceAB} promoter in
786 response to different levels of bacitracin and BceAB transporter. **(C)** Illustration of the *B.*
787 *subtilis* response to sudden bacitracin exposure. In the absence of bacitracin (*left panel*),
788 BceAB is inactive and BceRS remains in its default OFF state. Upon bacitracin exposure
789 (*middle panel*) high amounts of UPP-Bac complexes are formed and transport activity of
790 BceAB is high, triggering signalling via BceRS to induce *bceAB* expression (see also *red arrow*
791 in panel B). As a response, BceAB transporters accumulate in the cytoplasmic membrane
792 and remove bacitracin from UPP. This in turn reduces the flux experienced by individual
793 transporters, which ultimately reduces *bceAB* expression to an adapted steady-state level
794 (*right panel* and *green arrow* in panel B).

795

Figure 4. The periplasmic binding protein of the BctCAB transporter mediates transport as well as signalling in *Bordetella pertussis*. The citrate uptake system (*green*) of *B. pertussis* consists of the permease domains BctA and BctB, as well as the periplasmic binding protein BctC, which binds citrate (*purple*) and thus mediates citrate import via BctAB. In addition, BctC-citrate binds to and activates the histidine kinase BctE (*orange*), which in response stimulates expression of the *bctCAB* operon via the response regulator BctD. Hence, citrate binding to BctC increases the rate of transport and the magnitude of signal transduction in proportional ways, suggesting that the system might function akin to a positive flux sensor.

Figure 5. In the phosphate starvation response of *E. coli*, the 2CS PhoR-PhoB in conjunction with the $PstSCAB_2$ transporter functions as a negative flux sensor. Regulation of phosphate (*purple*) uptake in *E. coli* is controlled by a seven-protein signalling complex consisting of the phosphate transporter $PstSCAB_2$ (*green*), the 2CS PhoR-PhoB (*orange*) and the accessory membrane protein PhoU (*yellow*). (**A**) Under phosphate-replete conditions the $PstSCAB_2$ transporter is fully active and imports phosphate at high rate. In this state the transporter is believed to bring PhoU into a conformation that turns the bifunctional histidine kinase PhoR into a 'phosphatase ON' state. This leads to inactivation of the response regulator PhoB and thereby keeps transcription of the Pho regulon at a basal level. The functional domains of PhoR are shown as CA, catalytic domain; PAS, Per-Arnt-Sim domain; HIS, histidine-transfer and dimerization domain (please refer to text for details of information transfer and transport-repressing functions of PhoU). **(B)** Under phosphate-limiting conditions, $PstSCAB_2$ transporter activity decreases, leading to a weakening or loss in $PstSCAB_2$-PhoU-PhoR interaction. As a result, the kinase is de-repressed, causing

819 activation of signalling ('kinase on') and induction of *pstSCAB, phoU* and *phoBR* expression.
820 **(C)** Schematic illustration of the phosphate starvation response in *E. coli*. During phosphate
821 starvation, the drop in external and internal inorganic phosphate (P_i) leads to reduced P_i
822 influx via $PstSCAB_2$, which in turn triggers the production of further transporters (*phase II*).
823 The increasing number of transporters recovers the internal P_i to its initial level and
824 guarantees an adequate supply (*phase III*). Once external P_i levels return to high levels,
825 internal phosphate might transiently accumulate to high intracellular levels, because of the
826 high number of transporters still present (*phase IV*). Excessive phosphate uptake is
827 prevented by PhoU. Additionally, $PstSCAB_2$ restore repression of signalling and transporter
828 levels drop to pre-starvation levels by dilution during cell growth (*phase I and V*).

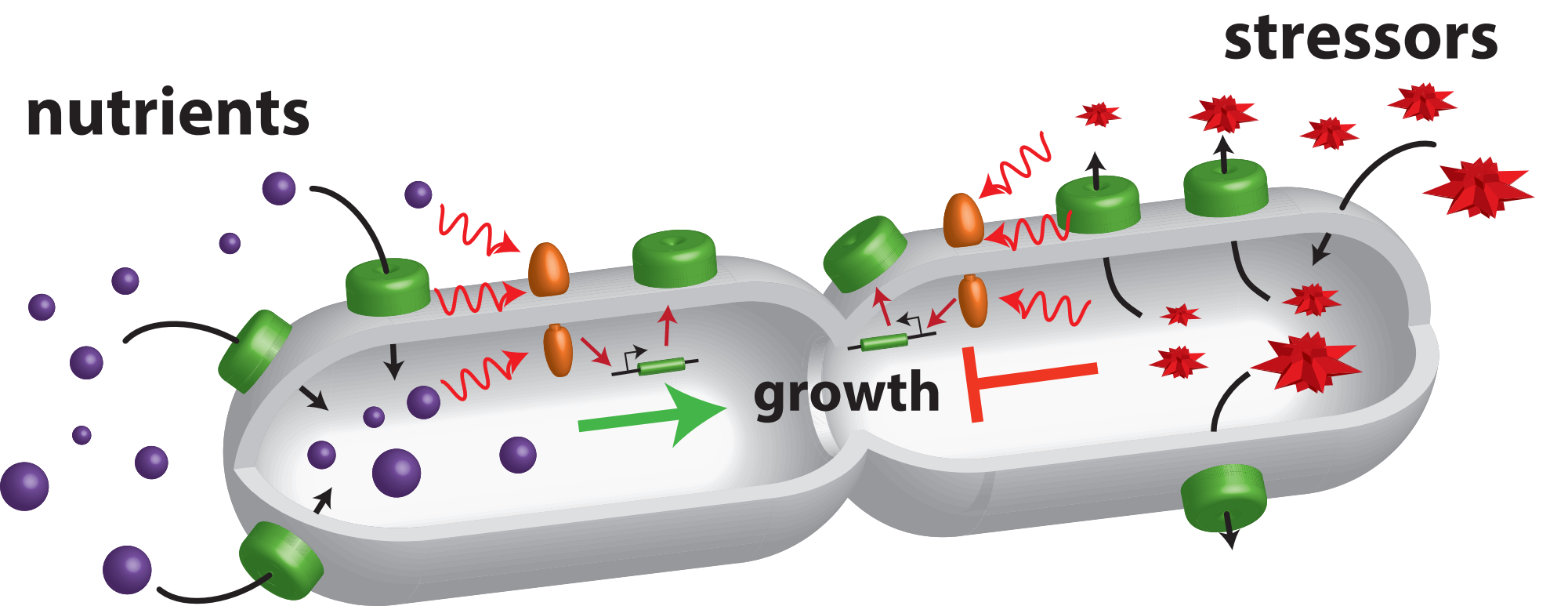
829

830 **Figure 6. The maltose transporter $MalEFGK_2$ of *E. coli* sequesters the transcriptional**
831 **regulator MalT at low transport flux.** In the maltose uptake system of *E. coli,* maltodextrins
832 (*purple*) are imported via the ABC transporter $MalEFGK_2$ (*green*). In the absence of external
833 maltodextrins, the inactive $MalEFGK_2$ transporter sequesters the transcriptional activator
834 MalT to the membrane (*left*). Upon maltodextrin import by $MalEFGK_2$, MalT is released to
835 the cytoplasm, where it is activated by binding of maltotriose (*light blue*) - a by-product of
836 maltodextrin metabolism produced by MalZ (*yellow*). Hence, after complete activation by
837 two signals – maltodextrin influx *and* internal maltotriose - MalT up-regulates the
838 expression of the transporter genes and others involved in maltodextrin metabolism.

839

840 **Figure 7. In the acid stress response of *E. coli*, the lysine importer LysP interacts with the**
841 **one-component system CadC to integrate three external signals. (A)** The acid stress

response system Cad of *E. coli* consists of the lysine decarboxylase CadA, the lysine/cadaverine antiporter CadB (*both yellow*) and the membrane-associated 1CS CadC (*orange*), which activates the expression of the *cadBA* operon at low pH, if simultaneously the lysine concentration is high and the cadaverine concentration is low. While CadC is able to sense the external pH and cadaverine directly via its periplasmic domain, the perception of external lysine relies on the interaction with the secondary lysine/H^+ symporter LysP (*green*). **(B)** Schematic illustration of the Cad-response to acid stress. In the absence of lysine (*left panel*), LysP inhibits the activation of CadC via intra-membrane and membrane-peripheral contacts. Under increased lysine levels *and* at low external pH (*middle panel*), high lysine influx via LysP releases this inhibition of CadC and allows the latter to be activated by low pH, resulting in the induction of *cadBA* expression. After the production of sufficiently high CadA and CadB levels (*right panel*), cadaverine is produced and excreted to the medium, where it increases the external pH and inhibits CadC, thereby terminating the Cad response.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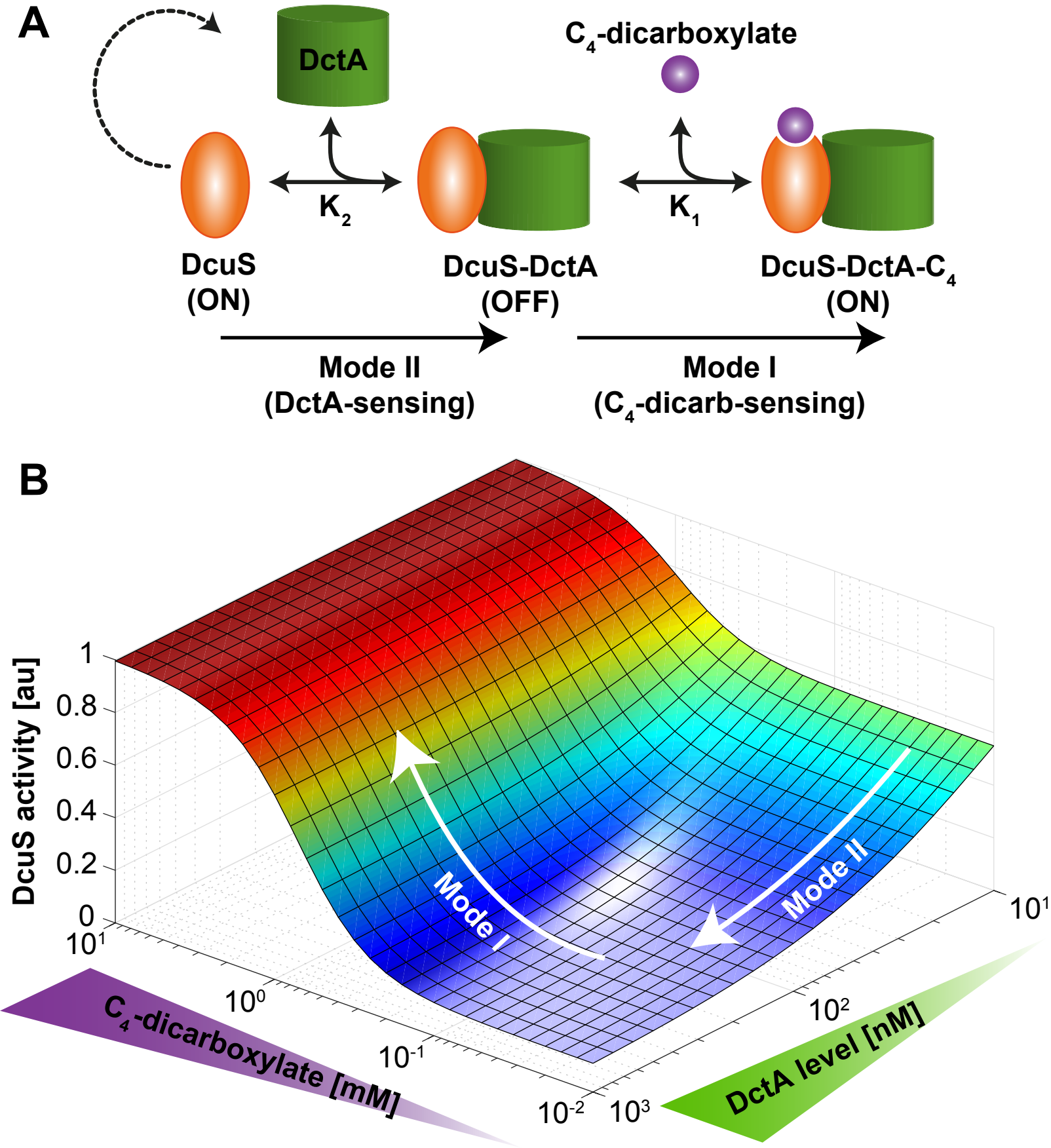
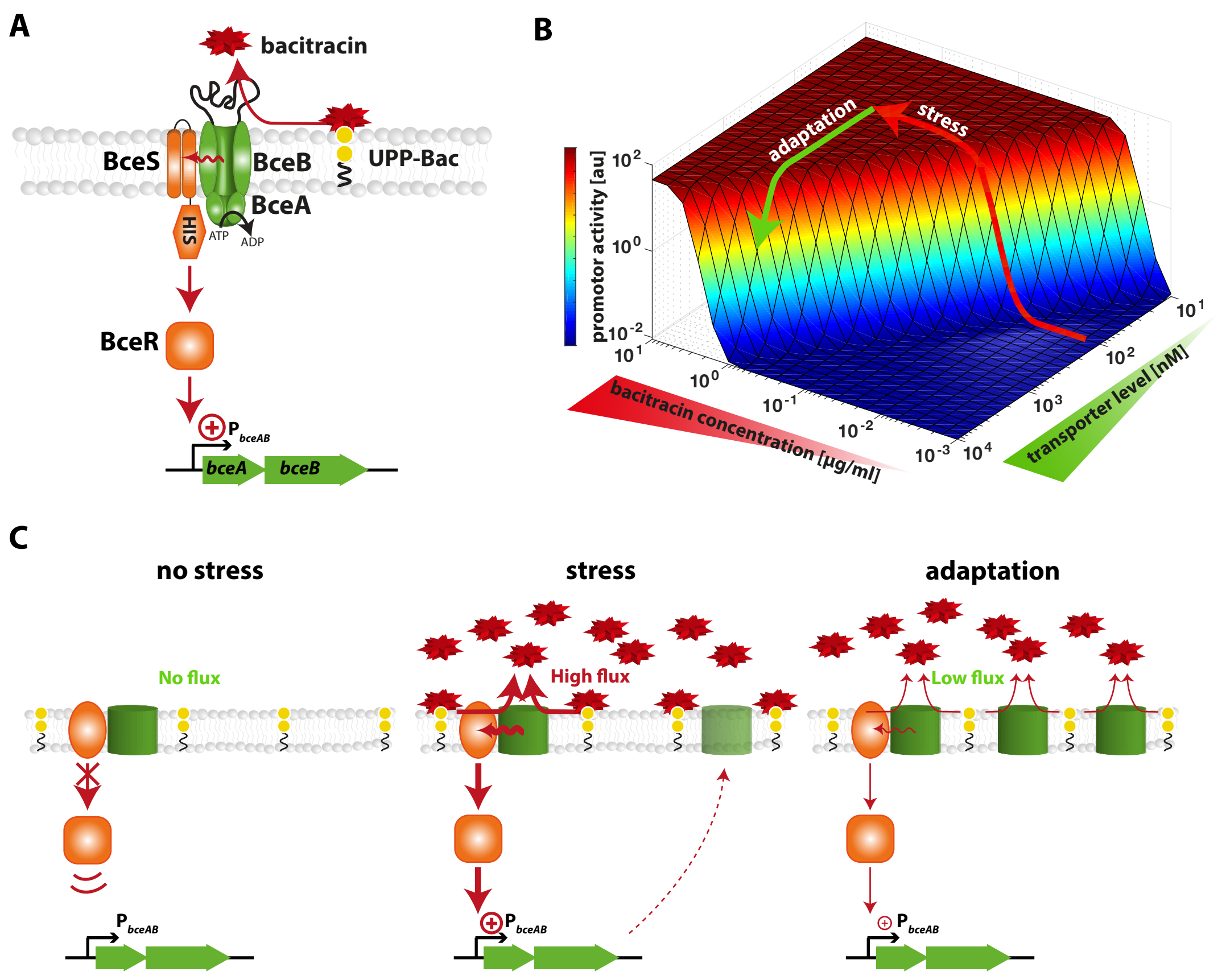
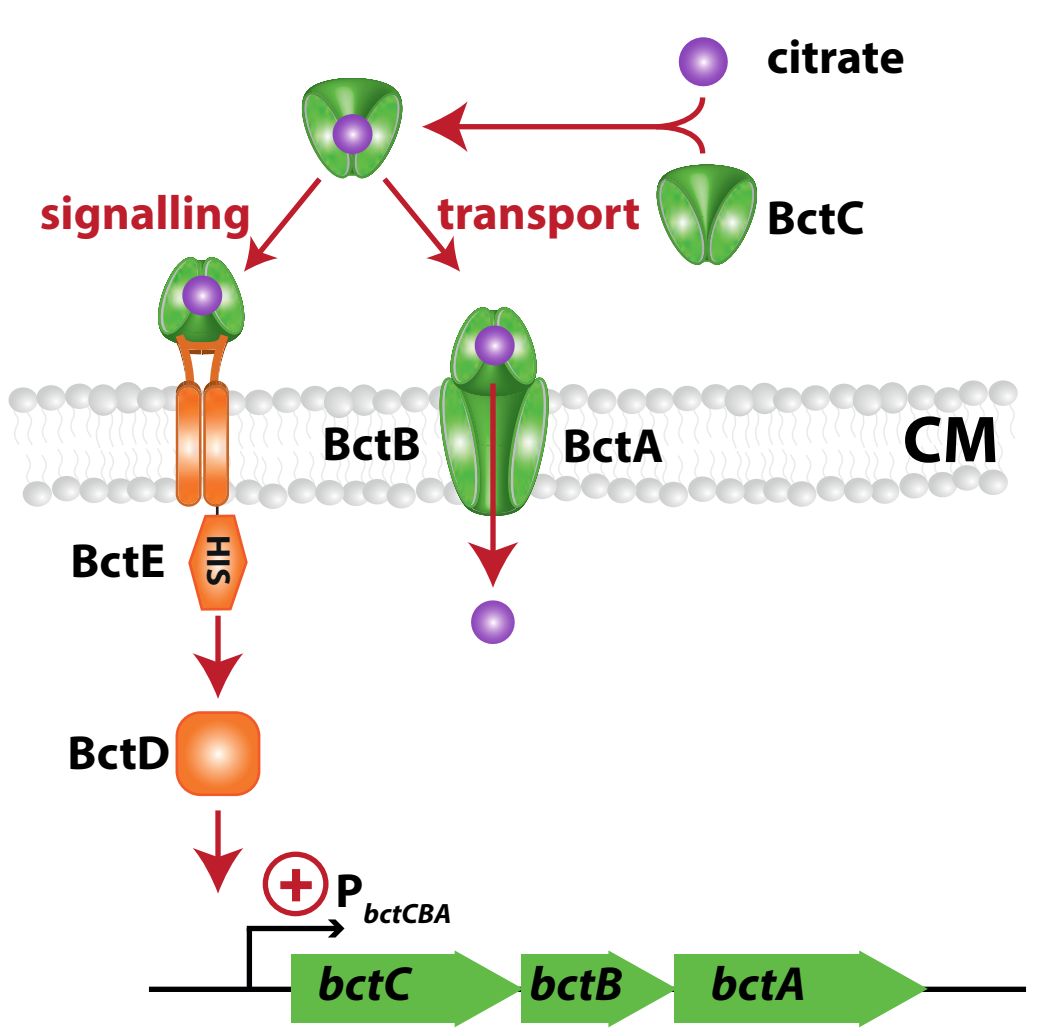
stressors
growth


A
DctA
C4-dicarboxylate
K2
K1
DcuS
(ON)
DcuS-DctA
(OFF)
DcuS-DctA-C4
(ON)
Mode II
(DctA-sensing)
Mode I
(C4-dicarb-sensing)
B
DcuS activity [au]
1
0.8
0.6
0.4
0.2
0
Mode I
Mode II
10^1
10^0
10^-1
10^-2
C4-dicarboxylate [mM]
10^1
10^2
10^3
DctA level [nM]


A
bacitracin
BceS
BceB
UPP-Bac
BceA
HIS
ATP
ADP
BceR
P bceAB
bceA
bceB
B
promotor activity [au]
10^2
10^0
10^-2
adaptation
stress
10^1
10^0
10^-1
10^-2
10^-3
10^4
10^3
10^2
10^1
bacitracin concentration [µg/ml]
transporter level [nM]
C
no stress
stress
adaptation
No flux
High flux
Low flux
P bceAB
P bceAB
P bceAB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BctC
signalling
transport
BctB
BctA
CM
BctE
HIS
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P bctCBA
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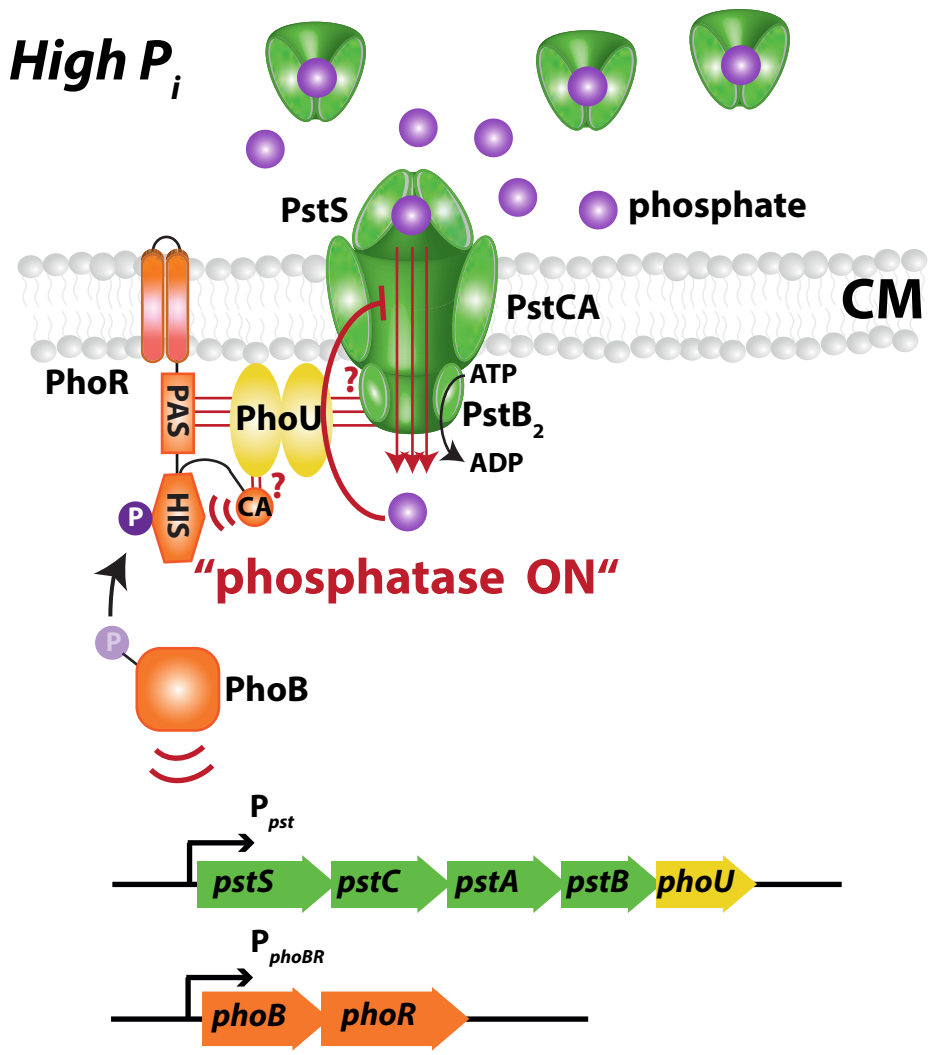
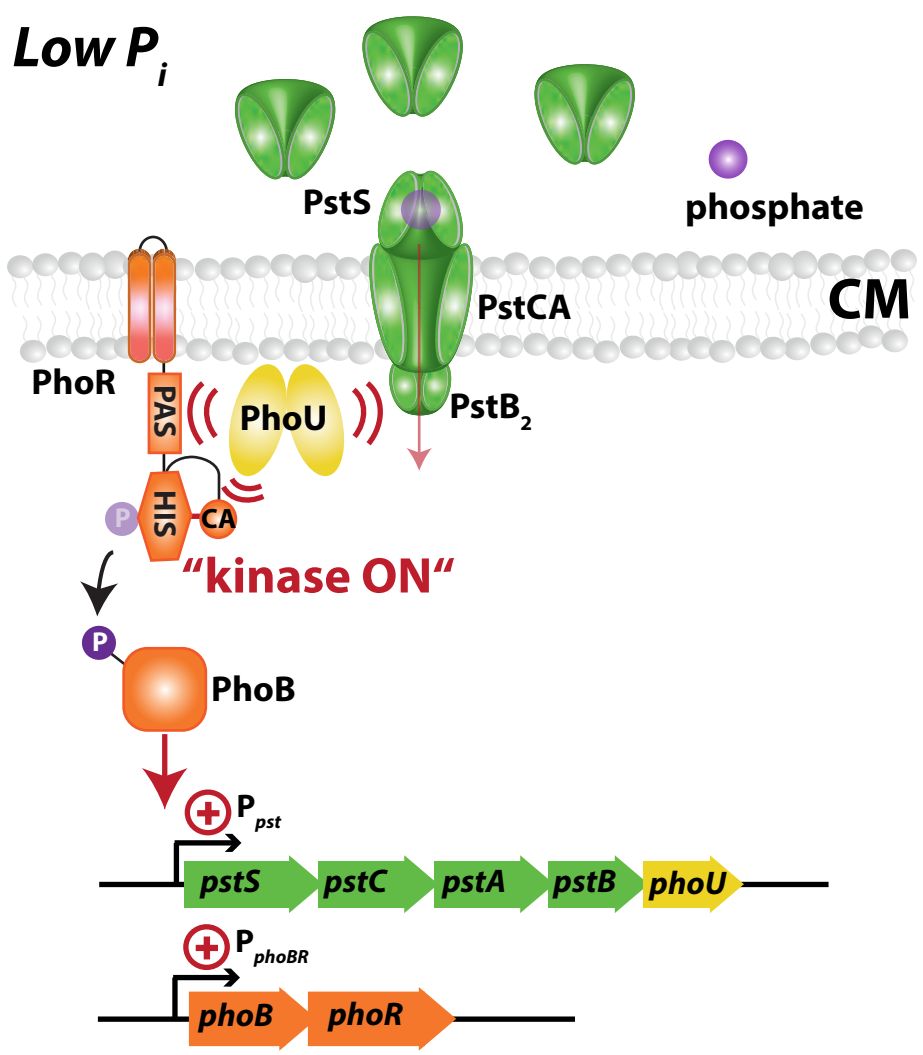
bctB
bctA

A


High P_i
PstS
phosphate
PstCA
CM
PhoR
PAS
PhoU
?
ATP
$PstB_2$
ADP
HIS
CA
?
P
"phosphatase ON"
PhoB
P_{pst}
pstS
pstC
pstA
pstB
phoU
P_{phoBR}
phoB
phoR

B


Low P_i
PstS
phosphate
PstCA
CM
PhoR
PAS
PhoU
$PstB_2$
HIS
CA
P
"kinase ON"
PhoB
P_{pst}
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pstC
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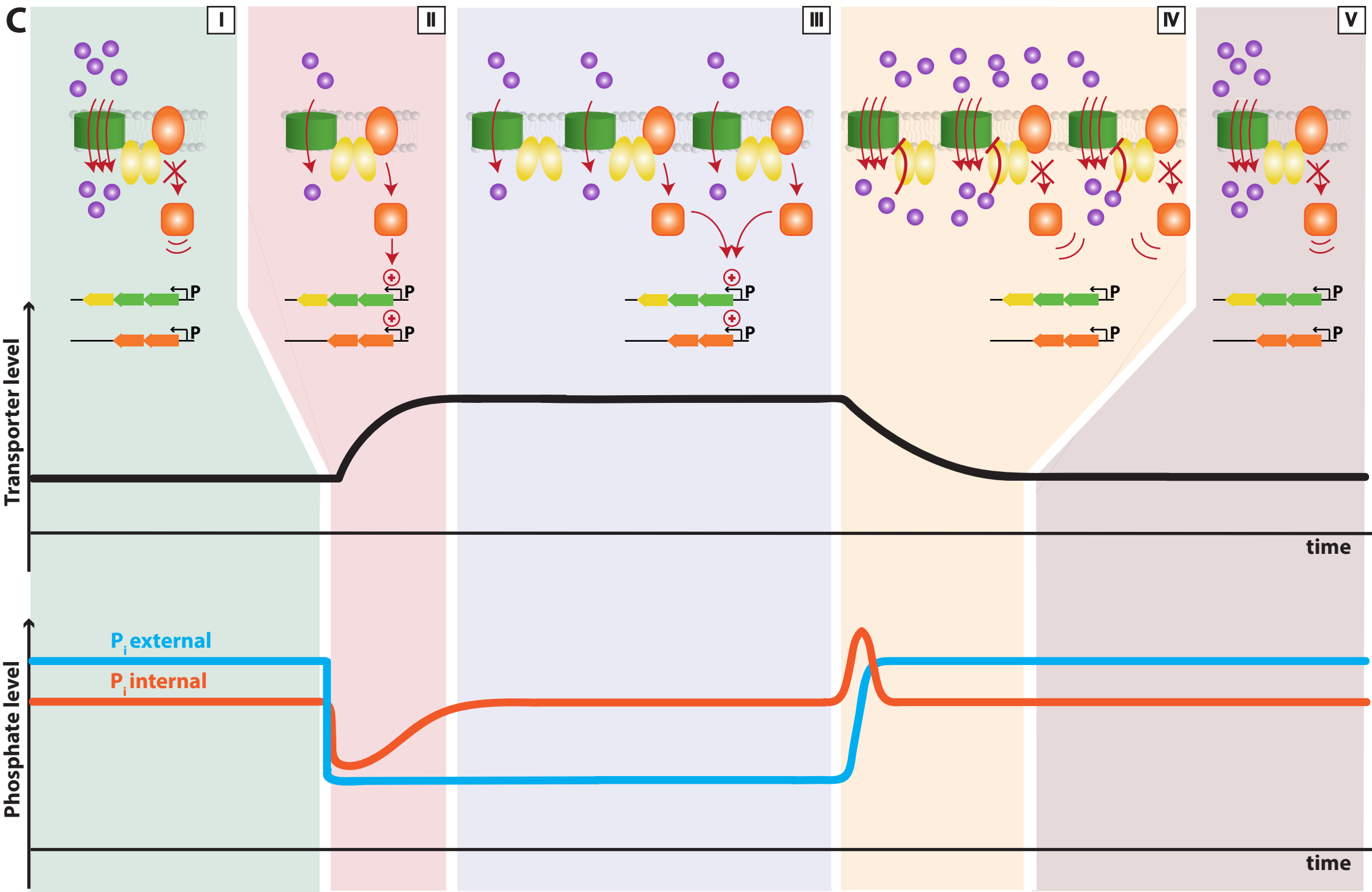
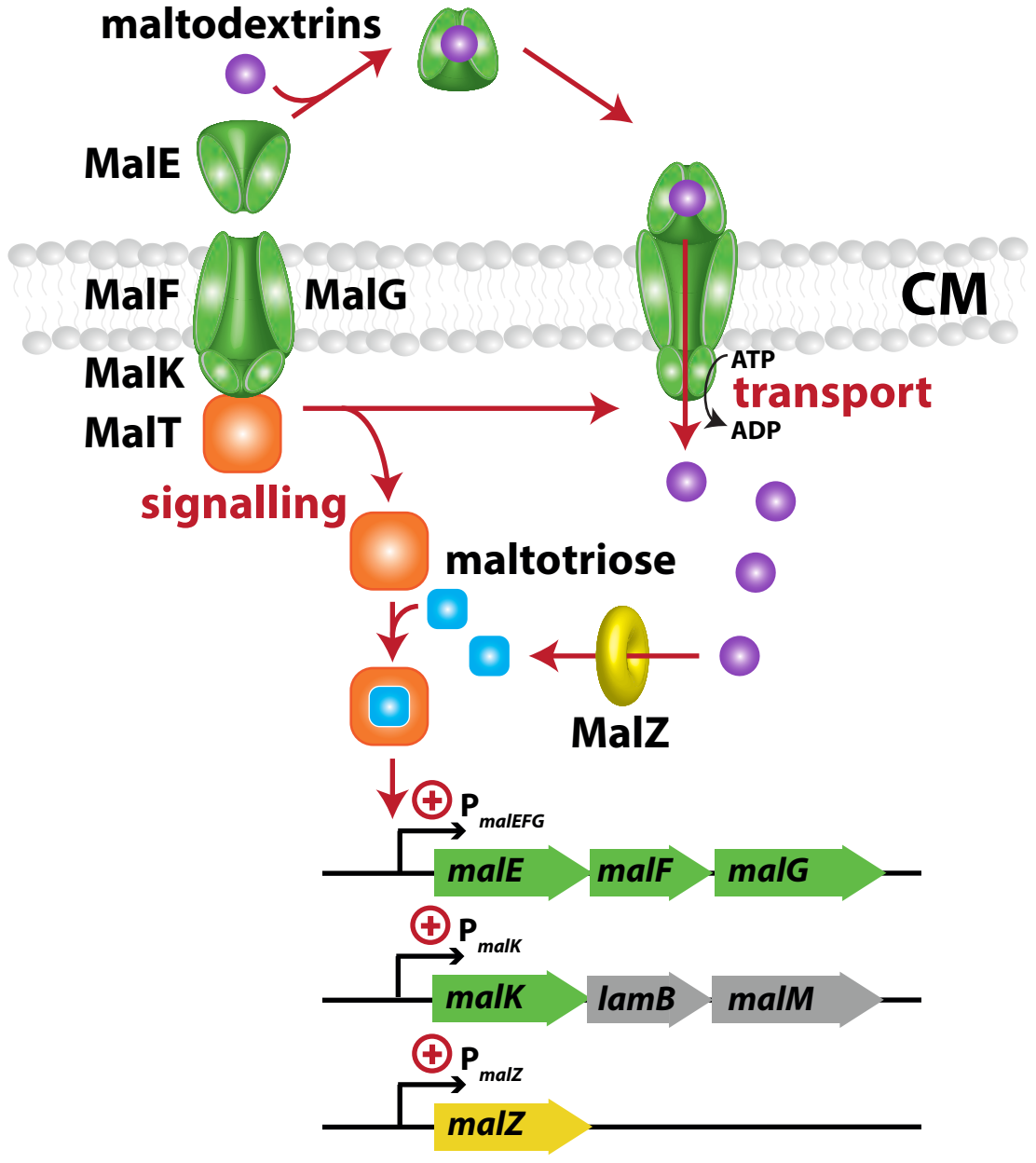
pstB
phoU
P_{phoBR}
phoB
phoR

C


I
II
III
IV
V
P
Transporter level
time
P_i external
P_i internal
Phosphate level
time


maltodextrins
MalE
MalF
MalG
MalK
MalT
CM
ATP
transport
ADP
signalling
maltotriose
MalZ
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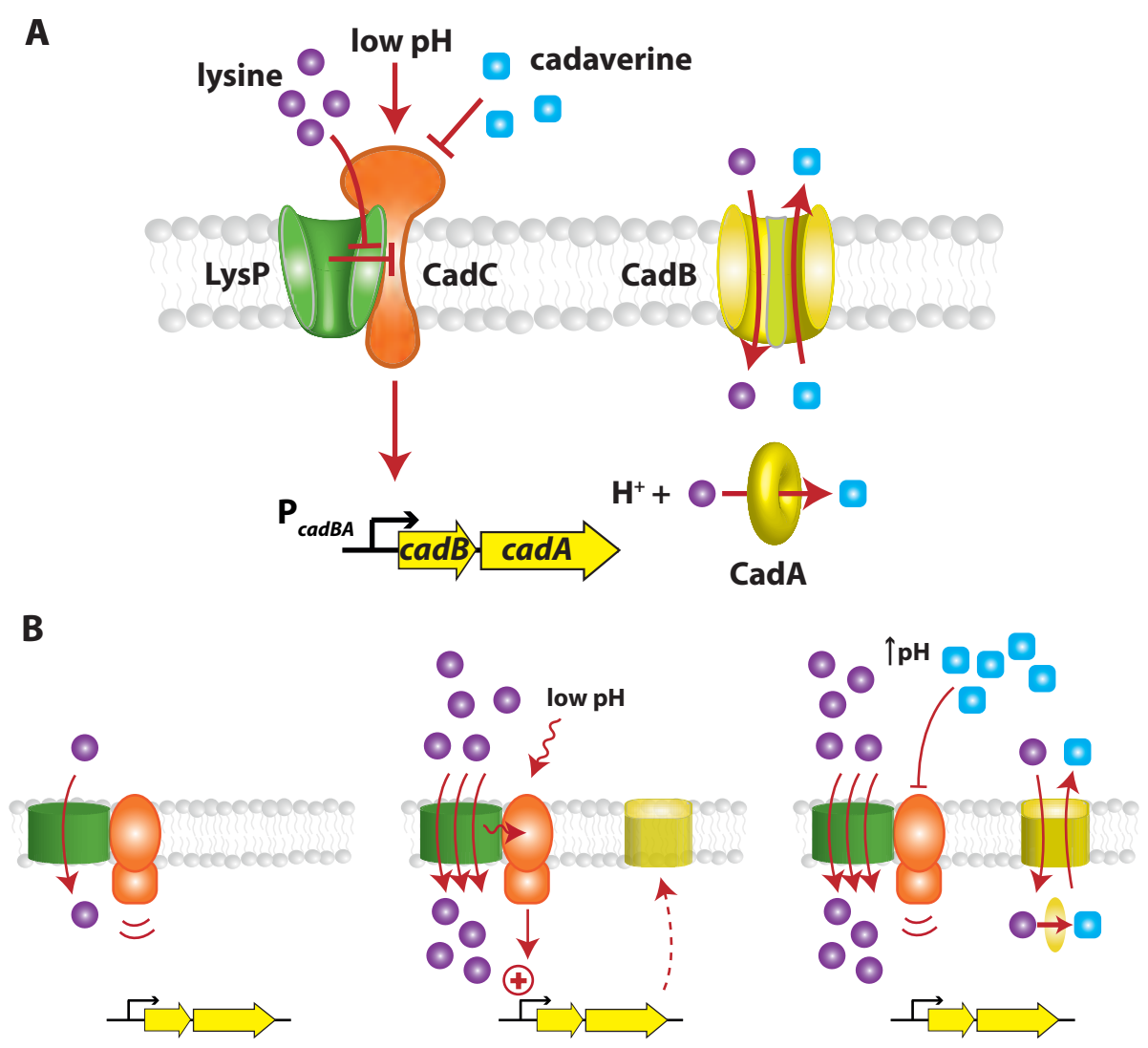
malE
malF
malG
P malK
malK
lamB
malM
P malZ
malZ


A
lysine
low pH
cadaverine
LysP
CadC
CadB
P cadBA
cadB
cadA
H+ +
CadA
B
low pH
↑pH